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Patterning and organization of motor neuron dendrites in the *Drosophila* larva

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ABSTRACT

Precise patterns of motor neuron connectivity depend on the proper establishment and positioning of the dendritic arbor. However, how different motor neurons orient their dendrites to selectively establish synaptic connectivity is not well understood. The *Drosophila* neuromuscular system provides a simple model to investigate the underlying organizational principles by which distinct subclasses of motor neurons orient their dendrites within the central neuropil. Here we used genetic mosaic techniques to characterize the diverse dendritic morphologies of individual motor neurons from five main nerve branches (ISN, ISNb, ISNd, SNa, and SNc) in the *Drosophila* larva. We found that motor neurons from different nerve branches project their dendrites to largely stereotyped mediolateral domains in the dorsal region of the neuropil providing full coverage of the receptive territory. Furthermore, dendrites from different motor neurons overlap extensively, regardless of subclass, suggesting that repulsive dendrite–dendrite interactions between motor neurons do not influence the mediolateral positioning of dendritic fields. The anatomical data in this study provide important information regarding how different subclasses of motor neurons organize their dendrites and establishes a foundation for the investigation of the mechanisms that control synaptic connectivity in the *Drosophila* motor circuit.

Introduction

The proper wiring of motor circuits during development is critical for normal locomotive behavior. Precise patterns of motor neuron connectivity depend on the selective connection between the axon and muscle target and the establishment of a unique dendrite arborization pattern that determines the specificity and degree of synaptic input. In the mouse and chick, spinal motor neurons are organized into distinct columns and pools and the positional identity of motor neurons correlates with the muscles they innervate (Jessell, 2000; Landmesser, 1978; Tsuchida et al., 1994). Motor neuron pools that target different muscle subsets establish distinct dendrite arborization patterns within the spinal cord and respond to sensory stimulation with different latencies (Vrieseling and Arber, 2006). Consequently, the selectivity of synaptic input is directly influenced by the differential orientation and positioning of motor dendrites in the spinal cord (Vrieseling and Arber, 2006). Therefore, understanding how different subclasses of motor neurons establish and position their diverse dendritic morphologies within the central nervous system (CNS) will be useful in deciphering how motor circuits are assembled.

Due in large part to the simplicity and morphological stereotypy of its neuromuscular system, *Drosophila melanogaster* has served as an invaluable tool in the study of motor circuit formation (Chisholm and Tessier-Lavigne, 1999; Collins and DiAntonio, 2007). In *Drosophila*, neurons and glia in the embryonic CNS are derived from progenitor cells called neuroblasts, which undergo multiple rounds of asymmetric cell division to generate a diversity of cell types (Goodman and Doe, 1993). Approximately 400 neurons, including an estimated 38 motor neurons, are generated from 30 distinct neuroblasts within each half-segment (or hemisegment) of the embryonic ventral nerve cord (VNC) (Schmid et al., 1999). Abdominal hemisegments in the embryo are comprised of 30 highly stereotyped body wall muscles, each of which is innervated by one or more of the 38 unique motor neurons (Landgraf et al., 1997). Motor axons exit the VNC through one of three main nerves (intersegmental nerve, segmental nerve, or transverse nerve) before innervating their specific target muscle(s) (Landgraf et al., 1997; Sink and Whitington, 1991). The intersegmental nerve is comprised of three nerve branches (ISN, ISNb, and ISNd) that target the internal muscles, while the segmental nerve is comprised of two nerve branches (SNa and SNc) that target the external muscles (Fig. 1). The origin and axonal projection patterns of the embryonic motor neurons that comprise the five main nerve branches have been well characterized and provide a reliable baseline for the identification and characterization of larval motor neurons in this study (Landgraf et al., 1997; Schmid et al., 1999).

Previous studies have shown that embryonic motor neurons with similar dendritic morphologies are generally derived from a common neuroblast and their axons innervate muscles that are functionally related (Landgraf et al., 1997). Unlike in the vertebrate spinal cord, the

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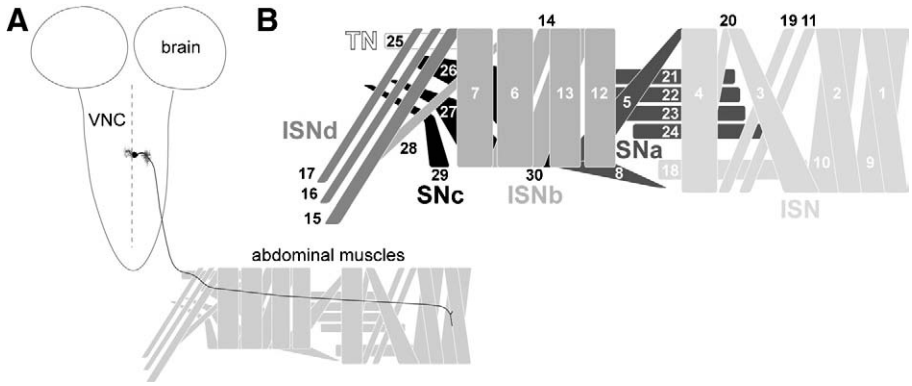

Fig. 1. The neuromuscular system of the *Drosophila* larva. (A) The larval CNS is comprised of the brain and ventral nerve cord (VNC), which is segmentally reiterated and bilaterally symmetrical with respect to the ventral midline (dotted line). A representative MARCM clone (MN9-Ib) is shown. (B) The stereotyped organization of the peripheral body wall muscles. In each abdominal hemisegment, motor axons from the six main nerve branches (ISN, ISNb, ISNd, SNa, SNc, and TN) innervate the 30 muscles.

selective connection between the motor axon and muscle target is not correlated with the cell body position of the neuron, but rather by the positioning of motor dendrites within the neuropil (Landgraf et al., 2003a). Dendritic fields of embryonic motor neurons are partitioned into distinct spatial domains within the VNC resulting in a myotopic map of the peripheral body wall muscles. This myotopic map is established independent of muscles and glia, suggesting that cell-autonomous mechanisms regulate the patterning and orientation of motor dendrites. In addition, dendrite–dendrite interactions between different motor neurons do not influence the organization of dendritic territories during embryogenesis (Landgraf et al., 2003a). However, the extent to which contacts between neighboring dendrites from the same or different subclass of motor neuron affects the establishment of dendritic fields during later developmental stages is not well known. Furthermore, it is unclear whether the spatial distribution of motor dendrites that forms the basis of the myotopic map is maintained during larval development.

By later larval stages, motor axon terminals are fully differentiated and neuromuscular synapses are well established and uniquely identifiable (Hoang and Chiba, 2001). However, there is a significant gap in our understanding of how motor dendrites establish their synaptic connections. A complete anatomical description of larval motor neurons is necessary to provide a foundation for the investigation of the mechanisms that control synaptic connectivity of motor circuits. In this study, we used mosaic analysis with a repressible cell marker (MARCM) to genetically label larval motor neurons with green fluorescent protein (GFP) to characterize their dendritic morphologies with single-cell resolution. We found that motor neurons elaborate largely stereotyped dendritic arbors that orient themselves within defined mediolateral domains in the central neuropil according to their corresponding nerve branch. Furthermore, dendrites from different motor neurons overlap extensively, suggesting cell-autonomous mechanisms control the mediolateral positioning of dendrites. The data in this study provide the first comprehensive description of larval motor neurons and provides further insight into the organizational principles of motor circuits in *Drosophila*.

Materials and methods

Generation of motor neuron clones

To generate motor neuron clones, we utilized the MARCM system (as described in Lee and Luo, 1999). *yw; FRT82B* flies were crossed to *elav-Gal4, UAS-mCD8::GFP, hs-FLP; FRT82B, tub-Gal80* flies. Clones were generated using *hs-FLP* (*hsp70 promoter-flippase*) to induce recombination at *FRT82B* and by visualizing mCD8::GFP expression driven by *elav-Gal4*, which drives Gal4 expression in all postmitotic neurons (Lin and Goodman, 1994). In brief, embryos were collected for 2 h and allowed to develop for 3–5 h at 25 °C before heat shock. Embryos were heat-shocked for 30 min at 38 °C, allowed to recover at room temperature for 30 min, then heat-shocked for an additional 45 min at 38 °C. Heat-shocked embryos were allowed to develop at 25 °C to third instar larval stage. Larvae with CNS clones were then selected before being dissected, fixed, and immuno-processed with the appropriate antibodies (see below). Fixed preparations were mounted on poly-L-lysine coated coverslips, dehydrated in an ethanol series, cleared in xylenes, and then mounted in DPX medium before imaging (Grueber et al., 2002).

Immunocytochemistry

Third instar larvae with motor neuron clones were immunolabeled with rat anti-mCD8 antibody at 1:200 dilution (Invitrogen, Carlsbad, CA) and monoclonal anti-Fasciclin II antibody (1D4) at 1:200 dilution (Developmental Studies Hybridoma Bank, University of Iowa). Cy2-and Rhodamine Red X (RRX)-conjugated secondary antibodies were used at 1:200 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA). Single-cell motor neuron clones were visually identified and confocal image stacks were obtained using a Leica TCS SP2 (Leica Microsystems, Bannockburn, IL) or an Olympus FluoView FV1000 (Olympus, Tokyo, Japan). Z-series stacks were reconstructed into single collapsed images using ImageJ software (National Institutes of Health, Bethesda, MD). Three-dimensional and orthogonal views were created using Volume Viewer in ImageJ.

Quantitative analysis

To quantify the average mediolateral position and width of the dendritic arbor, we plotted the relative positions of the most medial dendritic branch and most lateral dendritic branch with respect to the ventral midline and DL fascicle for each neuron using Adobe Photoshop (Adobe Systems Inc.). Because of the variability in the width of the neuropil for each preparation (between approximately 60–110 μm), values were normalized with the ventral midline position assigned a value of 0 and the DL fascicle position assigned a value of 1. Neurons that were rotated off-center with respect to the y-axis were not subjected to this analysis.

Results

Patterning of motor neuron dendrites in the larval CNS

To examine the dendritic arborization patterns of motor neurons in the larval CNS in detail, we used MARCM to genetically label single motor neuron clones with a membrane-targeted GFP (mCD8::GFP) (Lee and Luo, 1999). Although MARCM provides unparalleled single-

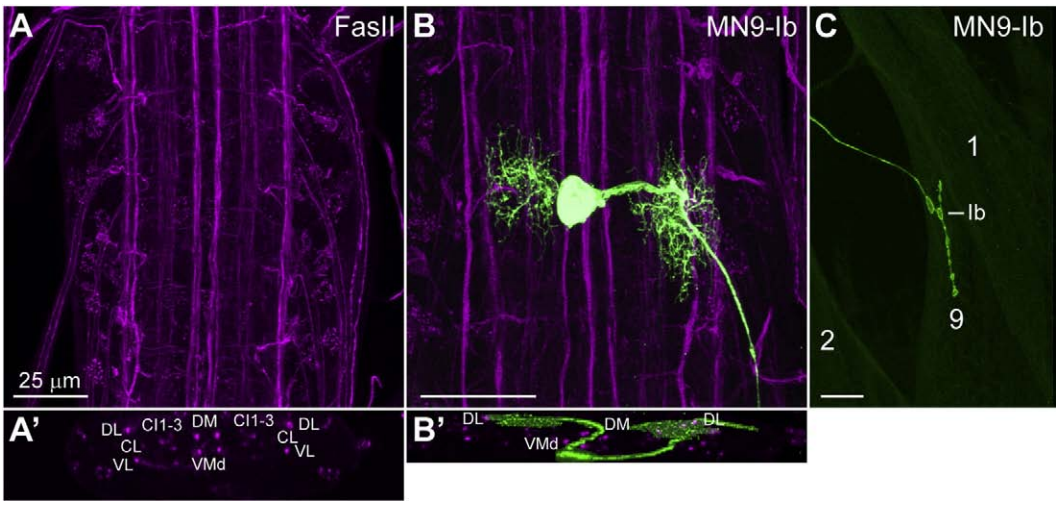

Fig. 2. Identification of individual motor neurons. (A) The stereotyped pattern of Fasciclin II expression in the VNC. Axon fascicles in the VNC provide a frame of reference to characterize the dorsal-ventral and mediolateral position of the motor neuron cell body. (A') Orthogonal view of (A). Fascicles are named according to Landgraf et al. (2003b) (D = dorsal, C = central, V = ventral, L = lateral, I = Intermediate). (B) Merged image of the GFP-labeled MN9-Ib motor neuron and FasII. (B') The soma of MN9-Ib resides in the ventral cortex, while its dendrites extend into the dorsal neuropil. (C) Peripheral muscle field innervated by MN9-Ib. MN9-Ib innervates muscle 9 and terminates with type Ib synaptic boutons. Anterior is up in this and all subsequent Figures.

cell resolution, some motor neurons may escape labeling due to low frequency of recombination in certain lineages. However, MARCM allows for the labeling of single motor neurons with axon terminals that are not easily accessible by conventional dye-backfilling methods such as DiI or Lucifer Yellow. Using MARCM, we unambiguously identified individual motor neurons according to the following criteria: (1) target muscle, (2) terminal synaptic bouton morphology, and (3) soma position (Fig. 2). Motor neurons were named by target muscle and bouton type according to the nomenclature described by Hoang and Chiba (2001) (Supplementary Fig. 1). We looked specifically at motor neurons whose axons terminated on peripheral muscles in abdominal segments A2–A6. In general, we found that the cell body

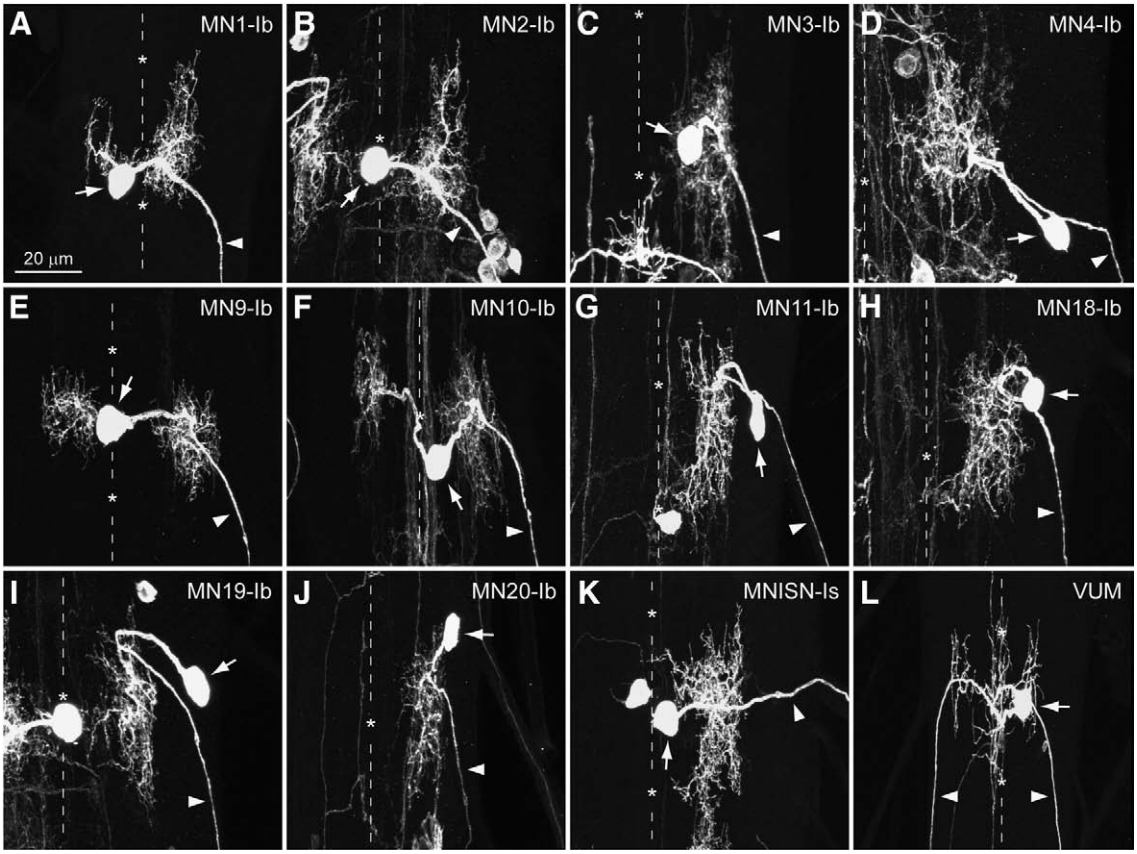

Fig. 3. Dendritic morphologies of motor neurons in the ISN branch. Single-cell motor neuron clones in the ISN branch. (A–L) Morphologies of individual motor neurons in the ISN branch. These neurons include MN1-Ib (A), MN2-Ib (B), MN3-Ib (C), MN4-Ib (D), MN9-Ib (E), MN10-Ib (F), MN11-Ib (G), MN18-Ib (H), MN19-Ib (I), MN20-Ib (J), MNISN-Is (K), and VUM (L). Both MNISN-Is and VUM innervate multiple dorsal muscles (not shown). The MN3-Ib neuron (C) is off-center. The soma of MN3-Ib is normally positioned in the lateral cortex. The dashed line represents the midline of the VNC, asterisks indicate position of segment borders. *Arrows* indicate position of cell body, *arrowheads* indicate axon.

positions of larval motor neurons are relatively fixed with respect to their embryonic counterparts. Furthermore, the basic dendrite arborization patterns of embryonic motor neurons are largely maintained throughout larval development. However, terminal dendritic branching is dramatically increased by the third instar larval stage and dendritic fields are significantly expanded within the central neuropil. The dendritic morphologies of individual motor neurons are largely stereotyped, with respect to position and orientation, from segment to segment as well as animal to animal facilitating the identification and characterization of these neurons (Supplementary Fig. 2). Motor neurons are classified according to their associated nerve branch and described in more detail below.

Intersegmental nerve (ISN)

ISN motor neurons

The ISN branch is comprised of the most morphologically diverse population of motor neurons among the five nerve branches characterized. We identified 12 unique motor neurons that project largely stereotyped dendritic arbors. MN1-Ib (Fig. 3A), MN9-Ib (Fig. 3E), and MN10-Ib (Fig. 3F), which innervate muscles 1, 9, and 10, respectively, are bipolar neurons that establish two distinct and highly branched populations of dendrites. Each motor neuron projects one arbor into the ipsilateral neuropil and a second arbor into the contralateral neuropil. Although morphologically similar, these motor neurons can be easily distinguished by their respective target muscles, terminal synaptic bouton morphologies, and cell body positions (Figs. 6B, C). MN2-Ib innervates muscle 2 and projects a single dendritic arbor into the ipsilateral neuropil (Fig. 3B). The cell bodies of MN3-Ib and MN4-Ib reside in lateral regions of the cortex and these motor neurons extend their axons and dendrites into dorsolateral regions of the neuropil before their axons exit into the periphery (Figs. 3C, D). Four motor neurons that innervate the dorsolateral muscles, MN11-Ib, MN18-Ib, MN19-Ib, and MN20-Ib, project their dendrites posteriorly within dorsolateral regions of the neuropil (Figs. 3G–J). We further identified a motor neuron that innervates multiple dorsal muscles (MNISN-Is), and projects its dendritic field into the ipsilateral neuropil (Fig. 3K). A ventral unpaired median (VUM) neuron projects its axons bilaterally to innervate multiple dorsal muscles of each hemisegment (Fig. 3L). All larval ISN motor neurons described here coincide with embryonically derived motor neurons (Table 1).

ISNb/ISNd motor neurons

Motor neurons in the ISNb branch that innervate the ventrolateral muscles, MN6/7-Ib (which innervates the cleft between muscles 6 and 7), MN12-Ib, MN13-Ib, MN14-Ib, and MN30-Ib, are unipolar and

Table 1
Embryonic origin of larval motor neurons.

Nerve	Embryonic motor neuron/neuroblast[a]	Target muscle(s)	Larval motor neuron[b] (*n*)	Target muscle(s)
ISN	aCC	1	MN1-Ib (12)	1
	U (NB7-1)	2	MN2-Ib (6)	2
	U (NB7-1)	3	MN3-Ib (1)	3
	U (NB7-1)	4	MN4-Ib (1)	4
	U (NB7-1)	9	MN9-Ib (24)	9
	U (NB7-1)	10	MN10-Ib (3)	10
	NB3-2	11	MN11-Ib (6)	11
	NB3-2	18	MN18-Ib (7)	18
	NB3-2	19	MN19-Ib (2)	19
	NB3-2	20	MN20-Ib (2)	20
	RP2	Dorsal	MNISN-Is (6)	1, 2, 3, 4, 9, 10, 18, 19, 20
	VUM-dorsal	Dorsal	VUM (3)	Dorsal
ISNb /ISNd	RP3	6, 7	MN6/7-Ib (4)	6, 7
	RP5 (NB3-1)	12	MN12-Ib (3)	12
	V (NB5-2)	12	MN12-III[c] (0)	12
	RP1 (NB3-1)	13	MN13-Ib (3)	13
	RP4 (NB3-1)	13	Not identified	
	NB4-2	14, 30	Not identified	
	Unknown		MN14-Ib (3)	14
	NB4-2	28	MN28-Ib (1)	28
	Unknown		MN30-Ib (6)	30
	NB7-1	15, 16	MN15/16-I (6)	15, 16
	NB7-1	17	Not identified	
	Unknown		MN15/16/17-Ib (3)	15, 16, 17
	Unknown		MNISNb/d-Is (4)	6, 7, 12, 13, 14, 15, 16, 20
	Unknown		MNISNb/d-II (4)	12, 13, 14, 15, 16, 17, 30
	VUM-ventral	Ventral	VUM (2)	Ventral
SNa	NB2-2	21	Not identified	
	NB2-2	22	Not identified	
	NB3-2	23	Not identified	
	NB3-2	24	Not identified	
	Unknown	5 and/or 8	MN5/8 (?)	
	Unknown		MN21/22-Ib (1)	21, 22
	Unknown		MN22/23-Ib (2)	22, 23
	Unknown		MN23/24-Ib[c] (0)	23, 24
	VUM-lateral	Lateral	VUM (1)	Lateral
SNc	NB4-2	26	Not identified	
	NB4-2	27	Not identified	
	NB4-2	29	Not identified	
	Unknown		MNSNc (5)	26, 27, 29

[a] Based on studies from Landgraf et al., 1997 and Schmid et al., 1999.
[b] Based on nomenclature from Hoang and Chiba, 2001.
[c] Not identified in this study.

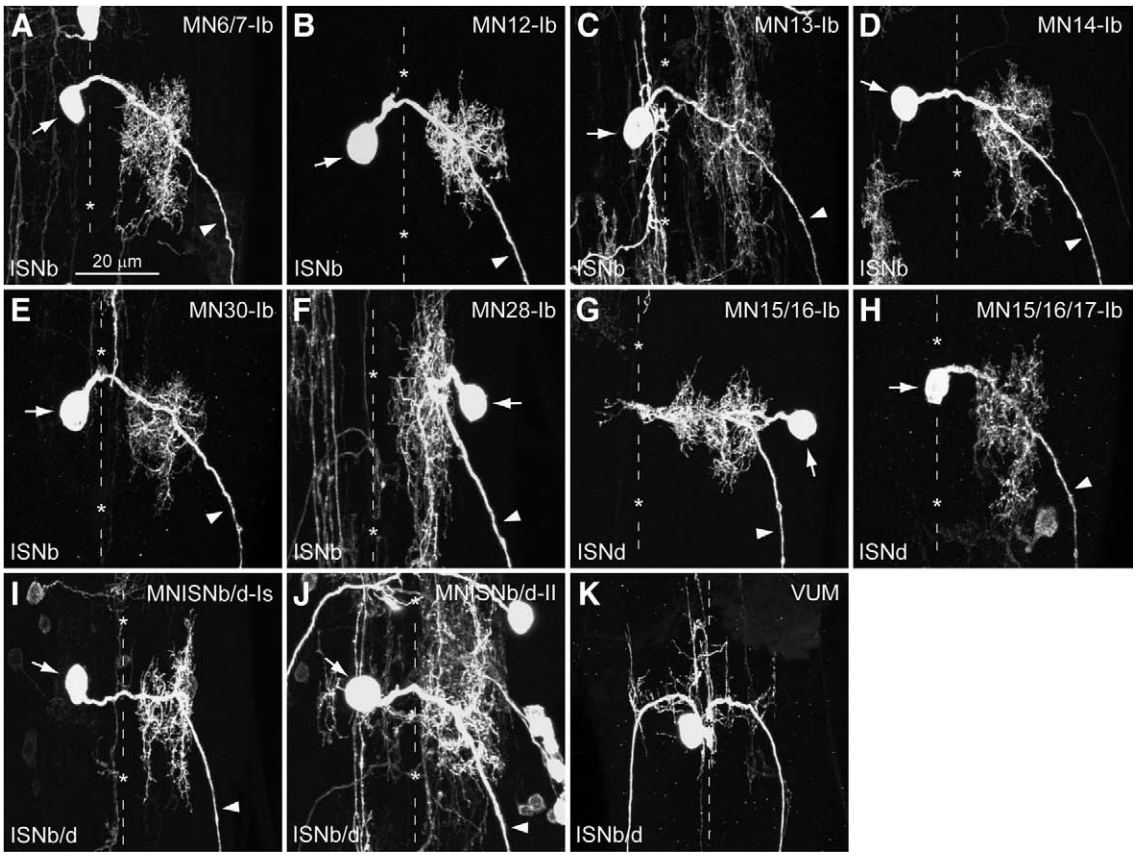

Fig. 4. Dendritic morphologies of motor neurons in the ISNb and ISNd branches. Single-cell motor neuron clones in the ISNb and ISNd branches. (A-F) Morphologies of individual motor neurons in the ISNb branch. These neurons include MN6/7-Ib (A), MN12-Ib (B), MN13-Ib (C), MN14-Ib (D), MN30-Ib (E), and MN28-Ib (F). (G, H) Morphologies of ISNd motor neurons MN15/16-Ib (G) and MN15/16/17-Ib (H). (I-K) ISNb/d motor neurons innervate muscles normally innervated by both ISNb and ISNd motor neurons including MNISNb/d-Is (I), MNISNb/d-II (J), and VUM (K). The dashed line represents the midline of the VNC, asterisks indicate position of segment borders. *Arrows* indicate position of cell body, *arrowheads* indicate axon.

project their highly branched dendritic arbors into the contralateral neuropil (Figs. 4A–E). MN6/7-Ib, which is normally bipolar in embryonic stages as the RP3 motor neuron, is either lacking or has few ipsilateral dendrites by the third instar larval stage in most cases observed (Fig. 4A). Although this motor neuron is maintained in the CNS from embryonic stages, it is possible that its dendrites are remodeled during larval development. Two distinct embryonic motor neurons target muscle 12 (Landgraf et al., 1997); however, we have thus far only identified a single larval motor neuron that innervates muscle 12 (MN12-Ib). We failed to detect the previously described MN12-III neuron using MARCM (Hoang and Chiba, 2001). Two embryonic motor neurons innervate muscle 13, though we concluded that MN13-Ib is the same neuron as the embryonic RP1 motor neuron based on morphology (Table 1). MN28-Ib innervates muscle 28 and its dendrites are morphologically distinct from other ISNb neurons which are derived from different neuroblasts (Fig. 4F; Table 1). The ISNd motor neuron MN15/16/17-Ib projects its dendrites into the ipsilateral neuropil (Fig. 4H), while the ISNd motor neuron MN15/16-Ib, which is unique from MN15/16/17-Ib, extends its axon and dendrites into the neuropil before the axon turns and targets the peripheral muscles (Fig. 4G). We could not identify a distinct embryonic origin of MN15/16/17-Ib (Table 1). Two different motor neurons that innervate multiple muscles, MNISNb/d-Is and MNISNb/d-II, are unipolar, project their arbors into the contralateral neuropil, and are morphologically similar to other ISNb motor neurons (Figs. 4I, J). These motor neurons do not have an identifiable embryonic counterpart. We also identified a VUM neuron that innervates multiple ventrolateral muscles normally targeted by ISNb motor neurons (Fig. 4K).

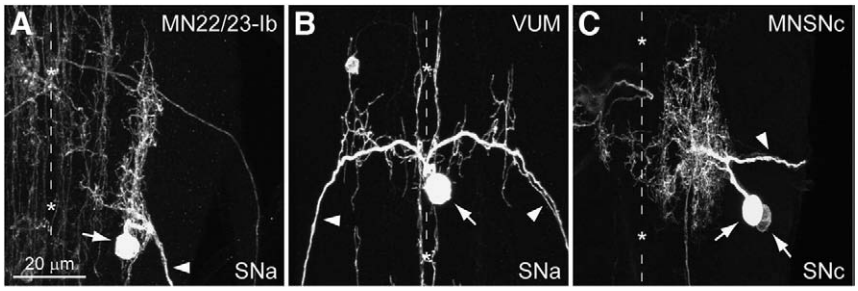

Fig. 5. Dendritic morphologies of motor neurons in the SNa and SNc branches. Single-cell motor neuron clones in the SNa and SNc branches. (A, B) Morphologies of individual motor neurons in the SNa branch including MN22/23-Ib (A) and VUM (B). (C) Dendritic morphology of MNSNc, which innervates multiple ventral muscles (not shown). The MNSNc motor neuron has two distinct cell bodies in MARCM clones. The dashed line represents the midline of the VNC, asterisks indicate position of segment borders. *Arrows* indicate position of cell body, *arrowheads* indicate axon.

Segmental nerve (SN)

SNa motor neurons

We identified two unique motor neurons that innervate the cleft between muscles 21/22 and 22/23. Both of these motor neurons have dendritic arbors that project anteriorly within dorsolateral regions of the neuropil (Fig. 5A and data not shown). Due to the limitations of MARCM, we were unable to identify a motor neuron that innervated muscles 23 and 24. We identified a single motor neuron that innervated either muscle 5 or muscle 8, but we were not able to unambiguously identify this particular neuron (data not shown). A single VUM neuron innervates the lateral muscles targeted by SNa motor neurons (Fig. 5B). Embryonic motor neurons of the SNa branch normally innervate single target muscles, so it is possible that SNa motor neurons remodel their axon terminals to target multiple muscles during larval development.

SNc motor neurons

A single SNc motor neuron that innervates multiple ventral muscles (26, 27, and 29) was newly identified in this study (MNSNc) (Supplementary Fig. 1V). We found that this particular neuron always had two cell bodies and is the only motor neuron

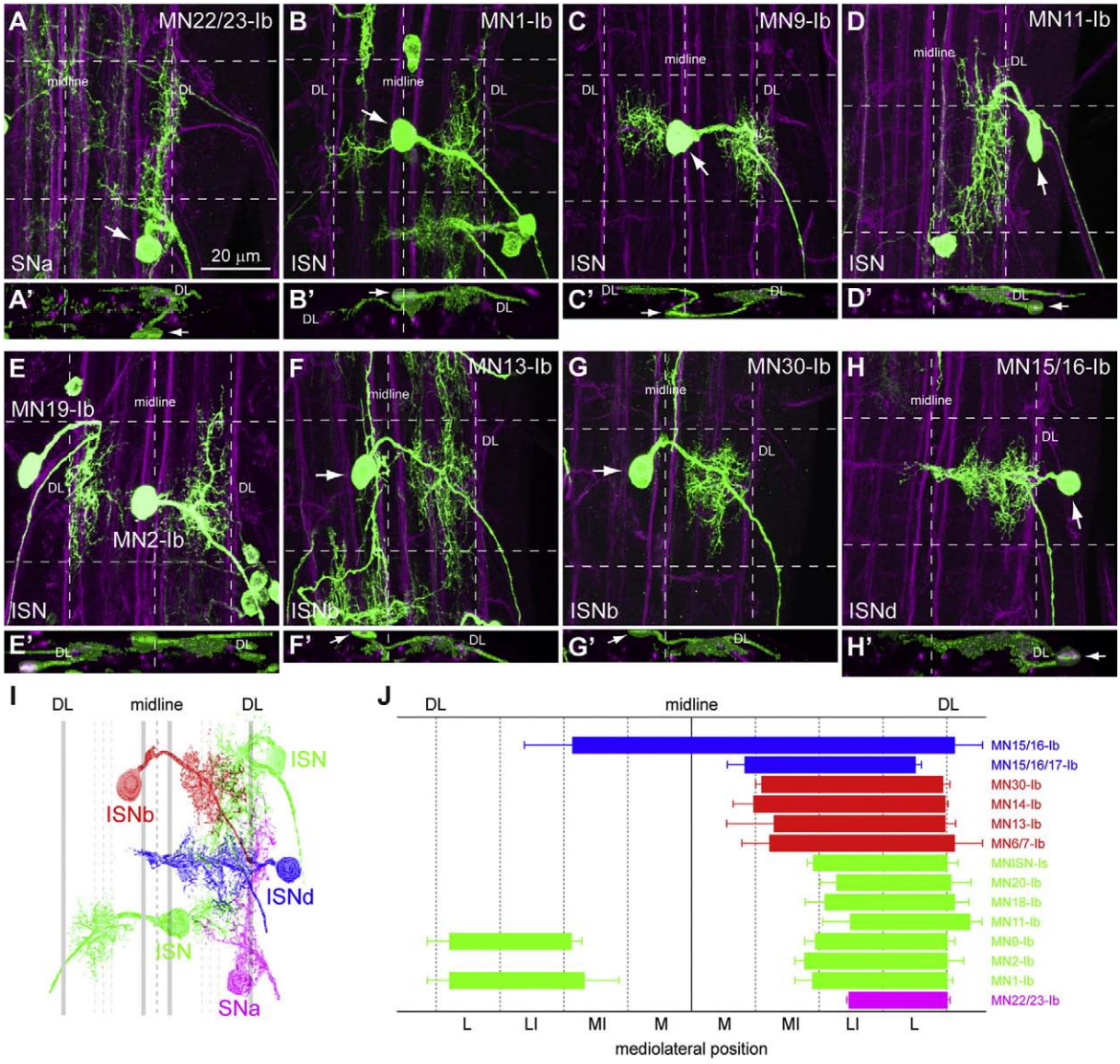

Fig. 6. Motor dendrites project to distinct neuropilar territories. The AP and mediolateral positioning of motor dendrites is largely stereotyped. (A, A') The cell body of the SNa motor neuron MN22/23-Ib resides in the ventrolateral cortex. MN22/23-Ib extends its axon and dendrite into the dorsolateral neuropil before its axon exits the VNC. MN22/23-Ib dendrites normally project anteriorly along the DL fascicle and extend slightly medial beyond this fascicle. (B, B') The cell body of the ISN motor neuron aCC is positioned within the dorsal cortex, and the motor neuron projects it dendrites to both ipsilateral and contralateral regions in the neuropil. Its dendritic field is normally restricted to the neuropil between the LI domain and DL fascicle. (C, C') The dendritic field positions of the ISN motor neuron MN9-Ib is similar to aCC, although the soma of MN9-Ib resides in the ventral cortex. (D, D') The cell body of the ISN motor neuron MN11-Ib resides in the lateral cortex. MN11-Ib extends its dendrites posteriorly between the LI domain and DL fascicle. (E, E') Both MN19-Ib and MN2-Ib dendrites project their dendrites between the LI domain and DL fascicle. (F, F') The cell body of the ISNb motor neuron MN13-Ib is located in the dorsal cortex, and the motor neuron extends its dendrites into the contralateral neuropil between the MI domain and DL fascicle. (G, G') The soma position and dendrite field of the ISNb motor neuron MN30-Ib is similar to MN13-Ib. (H, H') The ISNd motor neuron MN15/16-Ib is the only motor neuron that extends its dendrites to the ventral midline. (I) Schematic view of representative SNa (magenta), ISN (green), ISNb (red), and ISNd (blue) motor dendrites. Dendrites from motor neurons from each nerve branch occupy distinct territories to provide full coverage of the neuropil. (J) Average mediolateral positions and widths of representative SNa (magenta), ISN (green), ISNb (red), and ISNd (blue) motor dendrites relative to the ventral midline and DL fascicle. Lateral (L), lateral-intermediate (LI), medial-intermediate (MI), and medial (M) domains for each hemisegment are labeled. Values are normalized as described in Materials and methods and mean values ± standard deviation are shown. Vertical dashed lines represent the midline of the VNC and DL fascicle, horizontal dashed lines represent segment borders in A-H. *Arrows* indicate position of cell body, *arrowheads* indicate axon.

identified with this distinguishing feature (Fig. 5C; Supplementary Figs. 2F, F'). It is possible that the second cell body is the sibling of the MNSNc motor neuron. A routine failure of abscission during the terminal step of cytokinesis may produce two MNSNc cell bodies connected by an elongated intercellular bridge. However, there is currently no evidence of any embryonic SNc motor neuron with two cell bodies (Landgraf et al., 1997). Embryonic SNc motor neurons normally innervate single muscles, though we were unable to identify any individual motor neurons that innervated muscles 26, 27, or 29 (Table 1). However, the dendritic morphologies of these embryonic motor neurons are very similar to the larval MNSNc motor neuron, suggesting that one of these may remodel their axon terminals during larval development. Whether the other two embryonic SNc motor neurons target other muscles or undergo apoptosis is unclear.

Neuropil targeting specificity of dendritic fields

We next wanted to determine the logic by which motor neurons organize their dendritic fields in the neuropil. Previous studies have shown that motor dendrites form a myotopic map in the embryonic CNS, whereby dendritic fields are partitioned into distinct domains in the VNC that represent a spatial distribution of the body wall muscles in the periphery (Landgraf et al., 2003a). Although the anterior-posterior (AP) positioning of the dendrites represents the myotopic map and are largely invariant, the extent to which dendritic arbors from distinct motor neuron subclasses (or nerve branches) segregate laterally within the neuropil is not well defined. Such information will be critical to our understanding of connectivity by second-order neurons. To map the mediolateral positions of motor neuron dendrites in the neuropil, we co-immunostained our MARCM preparations with anti-Fasciclin II (FasII) antibody (Grenningloh et al., 1991). FasII labels axon fascicles that divide the neuropil into distinct territories and provides a frame of reference in which to map the dorsal-ventral and mediolateral positions of motor neuron dendrites (Figs. 2A, B) (Grueber et al., 2007; Landgraf et al., 2003b). Using FasII to define the position of the ventral midline and outermost dorsal–lateral (DL) axon fascicle, we plotted the normalized mediolateral position values of motor dendrites from different nerve branches relative to the midline and DL fascicle (see Materials and methods) (Fig. 6J). By dividing the neuropilar region between the midline and DL fascicle into four equivalent domains (lateral, lateral-intermediate, medial-intermediate, and medial), we found that motor neurons from different nerve branches target their dendrites to largely stereotyped mediolateral positions within the neuropil.

In general, we found that SNa motor neurons project their dendrites anteriorly along the DL fascicle and extend their dendrites midway into the lateral-intermediate domain of the neuropil (Figs. 6A, A', J). SNa motor dendrites never extend medially beyond this region. Dendrites of ISN motor neurons that innervate the most dorsal muscles (MN1-Ib, MN2-Ib, MN9-Ib, and MN10-Ib) span their ipsilateral arbors from the DL fascicle to the medial-most edge of the lateral-intermediate domain in most cases observed (Figs. 6B, B', C, C', E, E', J). ISN motor neurons with bipolar dendrites (e.g. MN1-Ib and MN9-Ib) typically project their contralateral dendrites from the DL fascicle to the lateral-intermediate domain, but occasionally extend their dendrites to territories within the medial-intermediate domain of the neuropil (Figs. 6B, B', C, C', J). ISN motor neurons that innervate the dorsolateral muscles also project their dendrites from the DL fascicle to the lateral-intermediate domain (Figs. 6D, D', E, E', J). On the other hand, we found that most ISNb motor neurons consistently project their contralateral dendrites to neuropilar territories between the medial-most edge of the medial-intermediate domain and the DL fascicle (Figs. 6F, F', G, G', J). However, of all motor dendrites observed, we found that only the ISNd motor neurons routinely target their dendrites into the medial domain. The ISNd motor neuron MN15/16/17-Ib projects its dendrites from within the medial domain to the DL fascicle (Fig. 6J), while the ISNd motor neuron MN15/16-Ib elaborates its dendritic field within the entire width of the neuropil between the midline and DL fascicle. MN15/16-Ib is the only motor neuron that extends its dendrites beyond the ventral midline into the contralateral neuropil (Figs. 6H, H', J).

In general, we found that SNa, ISN, ISNb, and ISNd motor neurons project their dendritic fields to progressively larger, overlapping mediolateral domains within each hemisegment, respectively, to provide full coverage of the receptive territories of the dorsal neuropil (Figs. 6I, J). The stereotyped mediolateral positioning of dendritic fields by different motor nerve branches may provide a mechanism by which synaptic connections are selectively formed. In addition to the AP positioning of dendrites in the myotopic map, this may then reflect a second layer of organization of the insect motor system.

Dendro-dendritic interactions do not influence the positioning of motor dendrites

Tiling, the complete and non-redundant coverage of a receptive field by dendrites of a single functional class, ensures proper segregation of synaptic or sensory inputs. Although specific classes of peripheral sensory neurons in *Drosophila* tile the body wall to organize their dendritic fields (Grueber et al., 2002), whether tiling affects the mediolateral positioning of *Drosophila* motor dendrites in the larval CNS is not well known. From our clonal analysis, we found that dendrites from different motor neurons overlap extensively (Figs. 7A–D), suggesting that repulsive dendrite–dendrite interactions between different motor neurons do not influence the formation of dendritic fields. However, this overlap of dendritic fields was evident regardless of whether motor neurons were from the same or different nerve branch (Figs. 7A, B), suggesting motor neurons within the same subclass do not exhibit “like-repels-like” interactions. Motor dendrites fail to reach the midline during larval development (Fig. 6), making it unlikely that tiling between homologous motor neurons influences the mediolateral positioning of dendrites. We further found

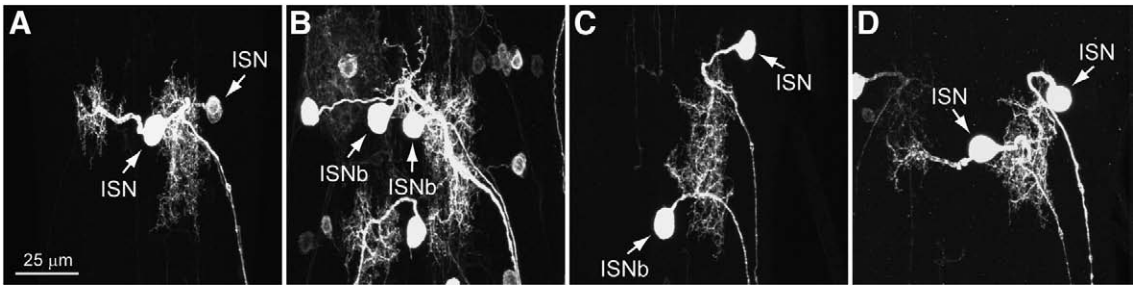

Fig. 7. Dendrites of different motor neurons show extensive overlap. (A, B) Dendrites from different motor neurons within the same nerve branch show extensive overlap. (A) Dendrites from two adjacent ISN motor neurons, MN9-Ib (left) and MN11-Ib (right), do not exhibit tiling behavior. (B) Dendrites from two adjacent ISNb motor neurons, MN6/7-Ib (left) and MN12-Ib (right), show extensive overlap. (C, D) Different motor neurons overlap with respect to segment boundaries regardless of whether they are from different nerve branches (C) or the same nerve branch (D).

that dendrites of different motor neurons in adjacent neuromeres overlap extensively, regardless of nerve branch (Figs. 7C, D). Therefore, repulsive dendro-dendritic interactions are unlikely to dictate either the AP or mediolateral positioning of motor dendrites during larval development.

Discussion

Embryonic origins of larval motor neurons

We have characterized the dendritic morphologies of individual motor neurons that comprise the ISN, ISNb, ISNd, SNa, and SNc nerve branches in the *Drosophila* larva. Using MARCM, we were able to visualize, with high resolution, different subcompartments (soma, dendrite, axon, and neuromuscular junction) of individual neurons allowing for the unambiguous identification of distinct motor neuron subtypes. Our data are largely consistent with previous studies identifying larval motor neurons based on terminal synaptic bouton morphology (Hoang and Chiba, 2001). Although some motor neurons target different and/or multiple muscles during the transition from embryo to larva, we found that most embryonic motor neurons are conserved in larvae with respect to soma position, primary dendrite morphology, and axon projection pattern. Based on these criteria, we find that the embryonic and larval forms of ISN motor neurons are likely the same (Table 1). However, consistent with previous reports, our results suggest that embryonic SNa and SNc motor neurons target different and/or multiple muscles in larvae (Hoang and Chiba, 2001). We were unable to identify any motor neurons that innervate single muscles normally targeted by either SNa or SNc motor neurons using MARCM. As embryonic motor neurons in the SNa and SNc branches are very similar morphologically to larval motor neurons, it is possible that many of these are maintained during larval stages. Which of these embryonic motor neurons persist into larval stages is still unclear.

Similar to segmental nerve motor neurons, we found that many of the embryonic origins of larval ISNb/ISNd motor neurons are not clearly defined (Table 1). The RP3 neuron, which innervates the cleft between muscles 6 and 7, is likely the same in embryos and larvae, although it is possible that its dendrites are remodeled during larval stages. On the other hand, muscles 12 and 13 are each innervated by two distinct motor neurons in embryos (Landgraf et al., 1997). Our results are consistent with previous studies that suggest the embryonic NB3-1-derived RP5 motor neuron is MN12-Ib (Hoang and Chiba, 2001). However, using MARCM, we were unable to identify MN12-III, which is thought to coincide with the NB5-2-derived V motor neuron (Hoang and Chiba, 2001). We identified a single larval motor neuron that innervates muscle 13 that is morphologically similar to the embryonic RP1 motor neuron that innervates muscle 13. The soma position and contralateral positioning of its dendrites are consistent with this particular neuron. We did not identify a larval motor neuron that correlated with the embryonic RP4 neuron in our MARCM studies. We also identified a larval motor neuron that innervates muscle 14 that is morphologically distinct from the NB4-2-derived embryonic motor neuron that innervates the cleft between muscles 14 and 30 (Table 1). Previous reports suggested that MNISNb/d-II is the mature state of the VUM-ventral motor neuron in embryos based on target muscle connectivity (Hoang and Chiba, 2001); however, we identified both MNISNb/d-II and VUM motor neurons in third instar larvae. Thus, the integration of our morphological data with previous studies is critical for the proper identification of the embryonic origins of larval motor neurons.

Mediolateral positioning of dendrites as a second layer of organization in the Drosophila motor circuit

How motor neurons elaborate their dendritic arbors to acquire their specific presynaptic inputs is a fundamental question of how neuronal circuits are formed. Recent studies show that the positioning and orientation of motor dendrites is critical for the selectivity of sensory-motor connections. In the mouse spinal cord, motor neuron pools that project their dendrites into the central grey matter respond to sensory stimulation with monosynaptic latency. In contrast, motor dendrites that avoid the central grey matter are activated through indirect connections (Vrieseling and Arber, 2006). In *Drosophila*, motor dendrites are confined to the dorsal region of the neuropil, suggesting that the proper AP and mediolateral positioning of dendrites within this dorsal region is critical for the proper receptivity of presynaptic inputs. While the central or AP positioning of embryonic motor dendrites defines the myotopic map (Landgraf et al., 2003a), the mediolateral positioning of dendrites within the neuropil may account for a second layer of organization among motor dendrites.

In general, we found that motor dendrites are partitioned into distinct, though largely overlapping, mediolateral territories based on the motor nerve branch (Fig. 6). This allows for full coverage of the dorsal region of the neuropil by dendrites of distinct subclasses of motor neurons. Although there is overlap among mediolateral dendritic territories, the morphological differences of motor neurons from different nerve branches are likely to further dictate the selectivity of synaptic input. Furthermore, as the VNC is segmentally reiterated, the stereotyped morphologies and orientations of motor dendrites in distinct mediolateral domains may facilitate synaptogenesis with ascending or descending longitudinal axons from second-order neurons (Rohrbough and Broadie, 2002), greatly reducing the complexity involved in establishing presynaptic connections. However, how these dendritic fields are established and defined within these distinct neuropilar territories is not well known.

We found that many dendritic fields are confined within territories demarcated by axon fascicles labeled with FasII. Therefore, the proximity of motor dendrites to these axon fascicles may provide clues as to how dendrite arbors are patterned. Although the AP positioning of motor dendrites is not dependent on glia (Landgraf et al., 2003a), midline glia may play a significant role in the mediolateral positioning of larval motor dendrites. In the developing embryo, a combinatorial code of Roundabout (Robo) receptors controls the mediolateral positioning of axons in the CNS in response to the chemorepellant Slit, which is produced by midline glia cells (Rajagopalan et al., 2000; Simpson et al., 2000). For example, ectopic expression of either Robo2 or Robo3 in axons that normally project medially causes a lateral shift in their position (Rajagopalan et al., 2000; Simpson et al., 2000). The Slit/Robo pathway also functions similarly in dendritic guidance. In the absence of *robo* function, dendrites of the embryonic RP3 motor neuron, which normally extend to more lateral regions of the ipsilateral and contralateral neuropil, often wrap around the ventral midline (Furrer et al., 2003). Dendrites of the embryonic aCC motor neuron are also shifted medially in *robo* mutants (Furrer et al., 2007). Furthermore, the transmembrane protein Commissureless (Comm) downregulates Robo expression and loss of *comm* causes a lateral shift in the position of the neuropil (Furrer et al., 2007; Keleman et al., 2002). In *comm* mutants, aCC dendrites shift laterally (Furrer et al., 2007), suggesting regulation of *comm*, and subsequently *robo*, are critical for proper positioning of dendrites. Recently, Mauss et al. (2009) reported that the combinatorial actions of Robo and Frazzled direct the mediolateral targeting of motor dendrites in embryos. Whether midline signaling systems are continuously required during larval development to maintain the mediolateral positioning of motor dendrites will be an active area of interest for future studies.

Repulsive dendro-dendritic interactions do not influence the organization of dendritic fields in the CNS

Tiling has been described in both mammalian and non-mammalian sensory systems as a general mechanism by which sensory neurons

organize their dendritic fields. In *Drosophila*, different classes of dendritic arborization (da) sensory neurons tile the larval body wall in a subtype-specific manner through "like-repels-like" interactions of neighboring dendrites (Grueber et al., 2002; Grueber et al., 2003; Sugimura et al., 2003). Although tiling is well established in sensory systems, the extent to which dendrite–dendrite interactions influence the formation of dendritic fields in the CNS is not well known. During larval development, dendritic fields of motor neurons expand considerably and refinement of dendritic territories may involve dendrite–dendrite interactions. However, our data suggest that repulsive interactions between dendrites of different motor neurons do not influence the formation or positioning of dendritic fields, regardless of whether the motor neurons are from the same or different subclass (or nerve branch) (Fig. 7A–D). Unlike *Drosophila* da neurons then, where different classes of neurons tile with respect to one another (Grueber et al., 2002), it seems that motor neurons do not adhere to strict rules of dendritic avoidance within distinct subclasses. With the exception of MN15/16-Ib, motor dendrites do not reach the ventral midline, suggesting that other cues, distinct from "like-repels-like" interactions, restrict their growth and influence their mediolateral positioning within the neuropil. However, this does not exclude the possibility of transient interactions at the midline during earlier stages of development which will need to be confirmed through time-lapse analysis.

In summary, the largely stereotyped nature of the dendritic arborization patterns of larval motor neurons in *Drosophila* provides a valuable model for the systematic analysis of how motor dendrites are established and patterned in the CNS. Molecular mechanisms that control dendritic guidance likely refine the territories of larval motor dendrites in the neuropil and will provide important insights into the assembly of motor circuits. Integrating data gathered from the embryonic and larval development of motor neurons will ultimately provide a foundation for the investigation of mechanisms that control synaptic connectivity, maintenance, and plasticity in the CNS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.041.

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